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I, GAYE TURNER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 0515 for a patent by PROTEOME SYSTEMS LTD filed on 29 September 2000.

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WITNESS my hand this
Twenty-fifth day of September 2001

Turner.

GAYE TURNER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

Patents Act 1990

Proteome Systems Ltd

PROVISIONAL SPECIFICATION

Invention Title:

Electrophoresis system

The invention is described in the following statement:

Field of the Invention

The present invention relates to a method for performing two-dimensional electrophoresis whereby both first and second dimension gels are contained within a single cassette.

Background of the Invention

For the last 25 years, 2-D PAGE has been the technique of choice for analysing the protein composition of a given cell type and for monitoring changes in gene activity through the quantitative and qualitative analysis of the thousands of proteins that orchestrate various cellular functions. Despite its extraordinary resolving power, 2-D PAGE has never been adopted for high throughput screening studies. This is because the first and second dimension gels are run separately, thus requiring two different running devices and making automation difficult. In addition, a high level of operator skill and knowledge is required to successfully complete a 2-D gel.

To achieve truly high throughput and reproducibility of 2-D gels it will be necessary remove as much of the operator intervention as possible. This could be accomplished by combining the first and second dimension gels in a single cassette and thus removing the need for the operator to interface the gels manually. This manual interfacing step is slow and cumbersome and a source of inter-operator differences thus leading to a lack of reproducibility. Another advantage of combining the two gels in a single cassette is the reduction of the running hardware to a single apparatus, thus increasing the possibilities of automation. Placing the gels together in a single cassette requires the ability to place the gels within a few millimetres of each other while maintaining the separation between the gels until the transfer from the first to the second dimension is required. There are serious technical difficulties associated with combining the first and second dimension gels in a single cassette. Firstly, the solutions present, and thus the ionic conditions, in the two gels are quite dissimilar. The first dimension, isoelectric focusing, gel has very low ionic strength and is not compatible with high levels of salts or buffers. Isoelectric focusing is performed at high voltage, up to 10,000 volts, and very low current, typically below 1mA per gel. Conversely the second dimension gel has a high concentration of buffer salts, usually in the 100 to 500mM range, and the electrophoresis is performed at high currents in the range of 5 to 100mA per gel. Thus, when placing these two gels in close proximity care must be taken to ensure that

there is no contamination of the first dimension gel with buffer from the second dimension gel, which would result in very high current and subsequent burning of the first dimension gel.

The second problem that one faces is the loading of the sample onto the first dimension gel without allowing any of the sample to prematurely transfer to the second dimension gel. The best way of performing isoelectric focusing is to use immobilised pH gradients (IPG), which are typically supplied as dry strips and rehydrated with the sample solution, thus distributing the sample over the entire gel, which allows high protein loads. When the two gels are combined in a single cassette it is difficult to wet the entire surface of the IPG with sample without allowing some sample to transfer to the second dimension gel.

Attempts have been made to provide combined strip gels and slab gels for carrying out 2-dimensional electrophoresis. US 4874490 discloses such an apparatus. However the proposed method is unwieldy and impractical and no examples of the method being carried out and actually working are given. With the recent upsurge in interest in the field of proteomics it becomes necessary to carry out many more separations than have previously been done. Separations, particularly two-dimensional separations are labour intensive and the only practical way of increasing the productivity of workers in the field is to provide methods and systems which are susceptible to automation. The method shown in US 4874490, if it works at all, would be difficult if not impossible to mechanise.

US 5773645 discloses a further apparatus for carrying out a two-dimensional separation on a common support. Again this specification does not disclose any examples or results from using the apparatus. Further the system disclosed would again be difficult and expensive to automate.

The present invention provides a new method which overcomes the technical difficulties associated with combining and running the first and second dimension gels in a single cassette in an manner which may allow the method and system to be automated.

Summary of the Invention

In a first broad aspect, it is the underlying idea of the present invention to propose a novel method for allowing a first dimension isoelectric focusing

gel and a second dimension electrophoresis gel to be combined, stored, shipped and run in a single cassette which is susceptible to automation.

In a second aspect the invention provides methods for rehydrating the first dimension gel with a solution of the sample proteins or polypeptides. Said rehydration occurs in the cassette without transfer of the sample solution to the second dimension gel.

In a third aspect the invention provides a method for placing a current insulating barrier between the first and second dimension gels while the isoelectric focusing is performed. The barrier prevents dehydration of both gels in the cassette and additionally prevents any premature transfer of sample from the first dimension gel to the second dimension gel or transfer of buffer salts from the second dimension gel to the first dimension gel.

Another aspect of the invention is a method for removing the current insulating barrier and providing a buffer impregnated bridging material, preferably a gel, to connect the first dimension gel with the second dimension gel. The bridging material allows proteins to transfer from the first dimension gel to the second dimension gel.

A further aspect of the invention is a means for providing electrical connections to the ends of the first dimension gel to allow the electrophoresis to proceed.

In a particular aspect there is provided a method for separating a sample into components by two-dimensional electrophoresis, said method comprising:

a. providing a first electrophoretic separation medium comprising an elongate strip typically an IPG strip and a second electrophoretic separation medium, typically in the form of a slab, said media being spaced apart and carried on a single generally planar support means;

b. with the planar support means oriented in a generally vertical plane and the first electrophoretic separation medium oriented in a horizontal plane spaced above or below the second electrophoretic separation medium by a gap, carrying out a first dimension separation of a sample mixture in the first electrophoretic separation medium, while the first and second media are separated by a non-conducting liquid which is substantially immiscible with water;

c. after the first separation has been carried out tilting the support means so that the first electrophoretic separation medium is at an angle to the

horizontal and flushing the liquid out from the gap between the first electrophoretic separation medium and the second electrophoretic separation medium; and

d. flowing a liquid buffer containing bridging material, typically agarose gel containing buffer, into the gap to allow transfer of sample molecules from the first electrophoretic separation medium to the second electrophoretic separation medium under the influence of an electric field.

It is preferred that the first electrophoretic separation medium is at least partly enclosed by a removable cover which allows the medium to be rehydrated, typically using a liquid containing the sample to be separated while the support means is in the vertical orientation.

The fact that the entire two dimensional electrophoresis separation may be carried out with the support means in the same vertical orientation facilitates the mechanisation of the system as compared with the prior art where the support means may be horizontally or both horizontally and vertically oriented.

The non-conducting liquid is most preferably paraffin oil.

Brief Description of the Drawings

Specific embodiments of the invention will now be described by way of example only and with reference to the accompanying drawings in which:-

Figure 1 is a schematic side view of a combined 2-D gel showing the IPG and second dimension gel positioned together in a single cassette ;

Figure 2 is a schematic top view of a combined 2-D gel showing the IPG and second dimension gel positioned together in a single cassette ;

Figure 3 shows two schematic side views of a single cassette plate prior to the assembly and casting of the second dimension gel. The IPG is attached to the cassette plate and is covered with a thin layer of plastic.

Figure 4 shows a fully assembled cassette as in Fig. 1 where the IPG is covered with a plastic cover sheet as in Fig. 3

Figure 5 shows a cassette as depicted in Fig. 4 where rehydration liquid has been introduced into the gap, containing the IPG, between the cassette wall and the plastic cover sheet.

Figure 6 is a schematic side view of a combined 2-D gel showing the positioning of the electrode bridge material and the electrodes at each end of the IPG gel

Figure 7 is a schematic top view of a combined 2-D gel showing the positioning of the electrode bridge material at each end of the IPG gel

Figure 8 is a schematic side view of a combined 2-D gel as in Fig. 3, where the cassette has been tilted to facilitate loading of the paraffin oil barrier material

Figure 9 is a silver stained two-dimensional gel run using the cassette arrangement described in Figs. 1 to 4;

Description of the Preferred Embodiments of the Invention and Examples

Referring to the drawings Figure 1 and 2 show the side and top views respectively of a combined 2-D gel 10 with a first dimension gel typically a dry immobilised pH gradient (IPG) type of gel 12, and a second dimension gel 14 positioned together in a single cassette 10. The cassette may be made of glass or plastic or any other suitable material. The IPG is generally spaced by a gap 16 of 2 mm away from the top surface of the second dimension gel and substantially parallel to the top surface of the second dimension gel. A gap 18 of approximately 5mm is maintained between the ends of the IPG gel and the side of the cassette to allow the insertion of electrode bridge material. Although the device shown in Fig.1 has a single IPG 12 placed parallel to the top of the second dimension gel and spaced at approximately 2mm from the second dimension gel, it will be clear to those skilled in the art that a combined 2-D gel of this design could be made to contain one or more IPGs in a linear array, spaced at more or less than 2mm from the top of the second dimension gel.

In a preferred embodiment the IPG is fixed to a single plate 20 of the cassette as shown in Fig. 3. The dry IPG is covered with a thin flexible plastic cover sheet 22, which is also fixed to the cassette plate to provide a liquid impermeable barrier, as shown in Fig. 3. The sheet may be made of other materials however they should be impermeable. The sheet may be conducting. The facing plate of the cassette, not shown, is subsequently attached and the second dimension gel 14 is cast within the cassette, thus producing the finished cassette as shown in Fig. 4.

To load the sample a small hole 24 is made in the IPG cover sheet and the liquid sample 26 is introduced into the gap surrounding the IPG, as shown in Fig. 5. It is important to introduce only enough liquid to rehydrate the IPG, as any liquid which is not absorbed will contain protein and thus

diminish the load. The dry IPG is allowed to rehydrate until all the liquid has been absorbed. After the rehydration process the IPG cover sheet is removed by peeling it away from the cassette using the protruding sample loading region 28 as a handle.

Alternatively but less advantageously it is possible to load the sample in a cassette as in Fig. 1 which does not have an IPG cover sheet. In this embodiment the cassette, as shown in Fig. 1, is placed horizontally as shown in Fig. 2, with the IPG gel facing up or down and rehydration liquid is carefully placed onto the surface of the IPG using a syringe. Capillary action holds the rehydration liquid onto the IPG surface and air acts as a barrier in the gap between the IPG and the second dimension gel, preventing the rehydration liquid from contacting the second dimension gel. Although the embodiment of the cassette shown in Fig. 1 shows uses air as the barrier between the gels during the rehydration process it will be clear to those skilled in the art that a combined 2-D gel of this design could use gases other than air, or liquids or solids as the barrier during the rehydration.

After the IPG gel has been rehydrated an electrode bridge must be placed in contact with each end of the IPG, as shown in Fig. 6 and 7. As shown in Fig. 7 the electrode bridge material 32 is held in place inside widened regions 30 of the cassette at each end. The electrode bridge material is commonly thick filter paper, cut to size and wetted with purified water. The advantage of having widened regions 30 is that larger thicker pieces of filter paper can be used as the bridge material. This is advantageous since during the application of an electric field to the gel the bridge material acts to soak up stray ionic material which is not wanted in the IPG. However, the electrode material could be made of any solid, inert, non-ionic, water absorptive material. In addition, in some cases it is desirable to soak the electrode material in buffers or sample solubilising solutions such as those used for IEF. For example, IEF solubilising solution may be used when highly solubilising conditions are required in the electrode bridge material to facilitate the removal of proteins which are not isoelectric within the IPG pH range. The electrode material acts as a reservoir between the gel and the electrode, where ionic contaminants and non-isoelectric proteins from the sample can collect without disrupting the separation. The advantage of having widened regions 30 is that larger thicker pieces of filter paper can be used as the bridge material which can soak up more stray ionic material. As

shown in Fig. 6 the electrodes 34 are usually pin shaped to allow penetration, and good contact, in the electrode bridge material.

In use,after the insertion of the electrode bridge material the gap between the IPG and the second dimension gel is filled with a barrier material to prevent dehydration of the IPG during the IEF step. In addition, the barrier prevents any transfer of liquid between the first and second dimension gels during the IEF. In a preferred embodiment the barrier material is light paraffin oil, however, it will be clear to those skilled in the art that paraffin oil with higher or lower density, or other oils may be suitable barrier materials. The defining characteristics of suitable barrier materials are water immiscibility, electrically insulating, chemically inert and preferably liquid at room temperature. The paraffin oil is inserted using a pipette with the cassette in a vertical position as shown in Fig. 8. The electrodes are placed into the electrode bridge material and the IEF is performed with the cassette in a vertical position.

To facilitate the transfer of protein from the IPG to the second dimension gel after the IEF the IPG requires equilibration with a suitable buffer. In a typical 2-D gel the equilibration buffer contains reducing agents such as dithiothreitol or tributyl phosphine and the surfactant Sodium Dodecyl Sulfate (SDS) which binds to the proteins and confers an overriding negative charge. The negative charge allows separation of the proteins according to molecular weight in the second dimension. In a preferred embodiment of this invention the IPG equilibration and SDS binding is done by displacing the paraffin oil barrier with a solution of molten 0.5% agarose containing SDS, glycerol and the appropriate buffer. In the preferred embodiment the proteins are reduced and alkylated prior to the first dimension separation. However, if the proteins have been separated in their reduced forms in the first dimension a reducing agent such as dithiothreitol or tributyl phosphine is added to the agarose. The molten agarose (typically 1-5 mL) solution is flushed through the gap between the first and second dimension gels, thus thoroughly removing the paraffin oil. Finally molten agarose containing SDS, glycerol and buffer is allowed to set in the gap between the first and second dimension gels, thus bridging the gap and allowing for protein transfer. To facilitate the addition of the hot agarose it is essential to have the cassette in a tilted vertical position as shown in Fig. 8. When the air bubbles have been flushed out of the gap the cassette is

returned to a normal vertical position as in Fig. 6 and allowed to stand at room temperature for 20 minutes to allow the agarose to set and the TBP and SDS to diffuse into the IPG. The standing time may be varied from zero to more than 20 minutes depending on the sample and the protein load. This system is particularly preferred as it is simple and lends itself to automation.

In a second less preferred, embodiment of the current invention a conventional equilibration solution, containing urea, SDS, reducing agents, buffer and glycerol can be flushed through the gap, displacing the paraffin barrier. This equilibration solution is allowed to remain in the cassette for between 5 minutes and 30 minutes and is usually changed up to 10 times, however it will be clear to those skilled in the art that the equilibration time and the number of changes may be varied without departing from the spirit of the invention or damaging the resulting gel.

The running of the second dimension gel and the subsequent staining are performed according to normal procedures. Figure 9 shows a silver stained 2-D gel of Rat liver run in a cassette as shown in Fig. 1. It is clear that the rehydration and IEF steps have worked well and the barrier materials used have prevented any premature transfer of the sample to the second dimension.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty-ninth day of September 2000

Proteome Systems Ltd
Patent Attorneys for the Applicant:

F B RICE & CO

Figure 1

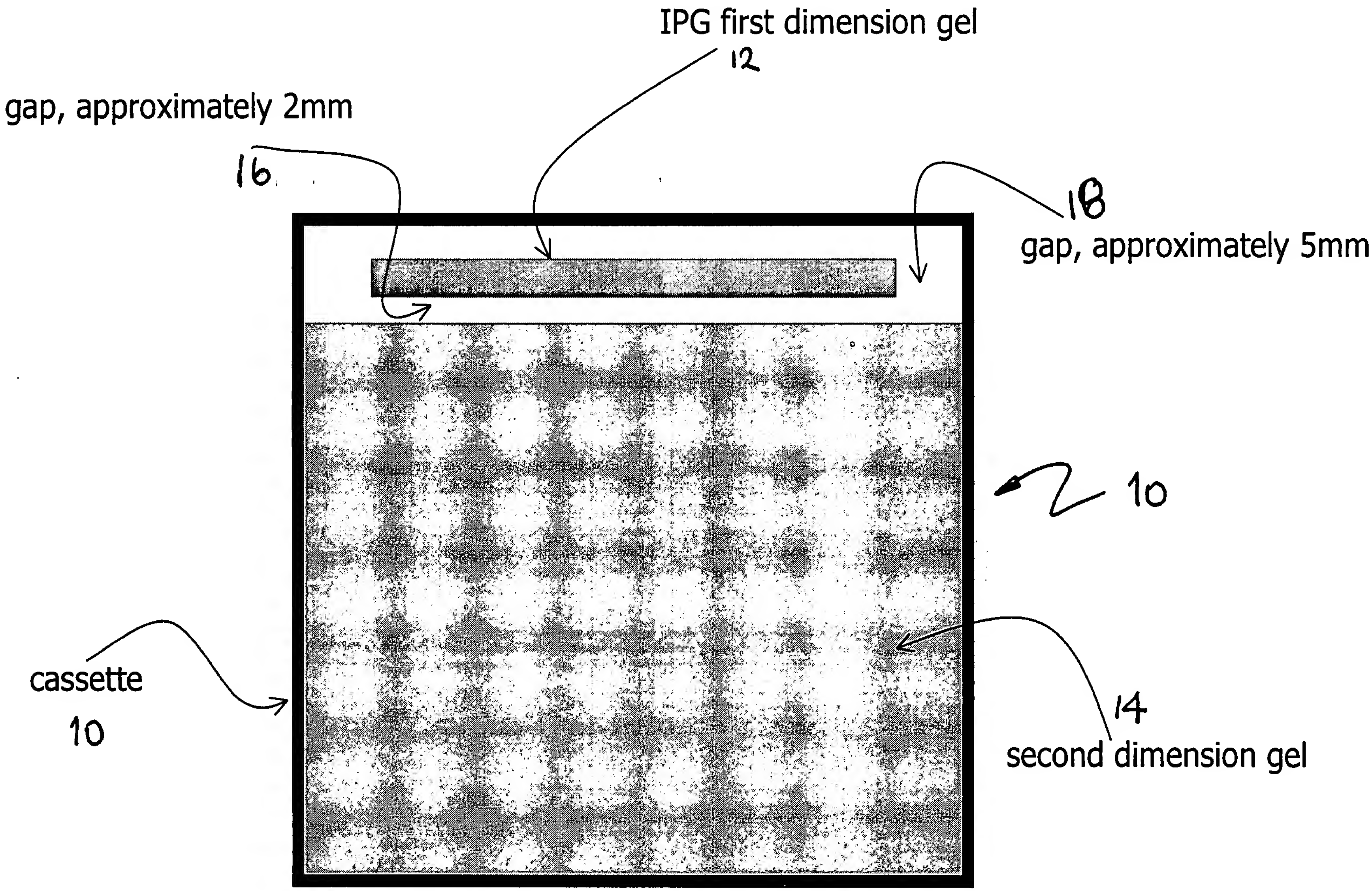
IPG first dimension gel
12
gap, approximately 2mm
16
18
gap, approximately 5mm
10
cassette
10
14
second dimension gel

Figure 2

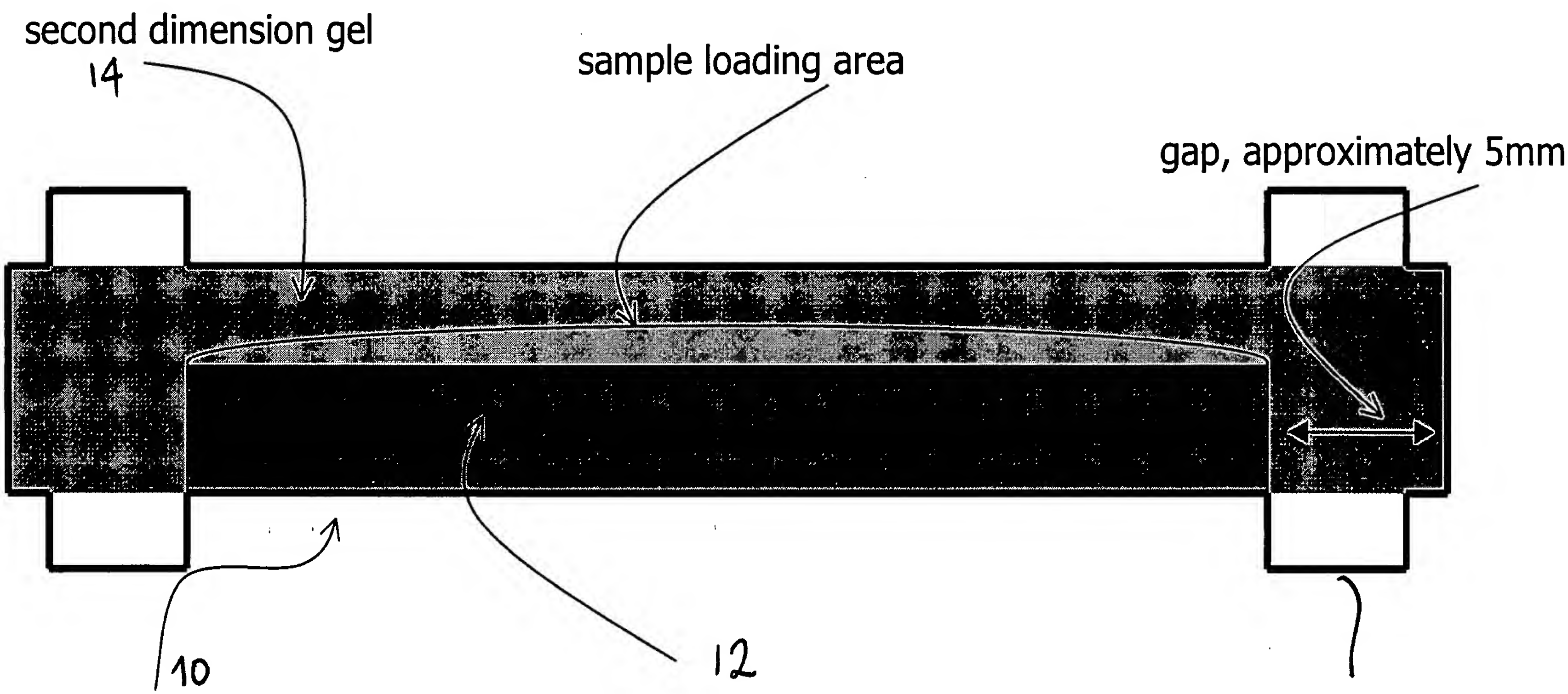
second dimension gel
14
sample loading area
gap, approximately 5mm
10
cassette
12
IPG first dimension gel

Figure 3

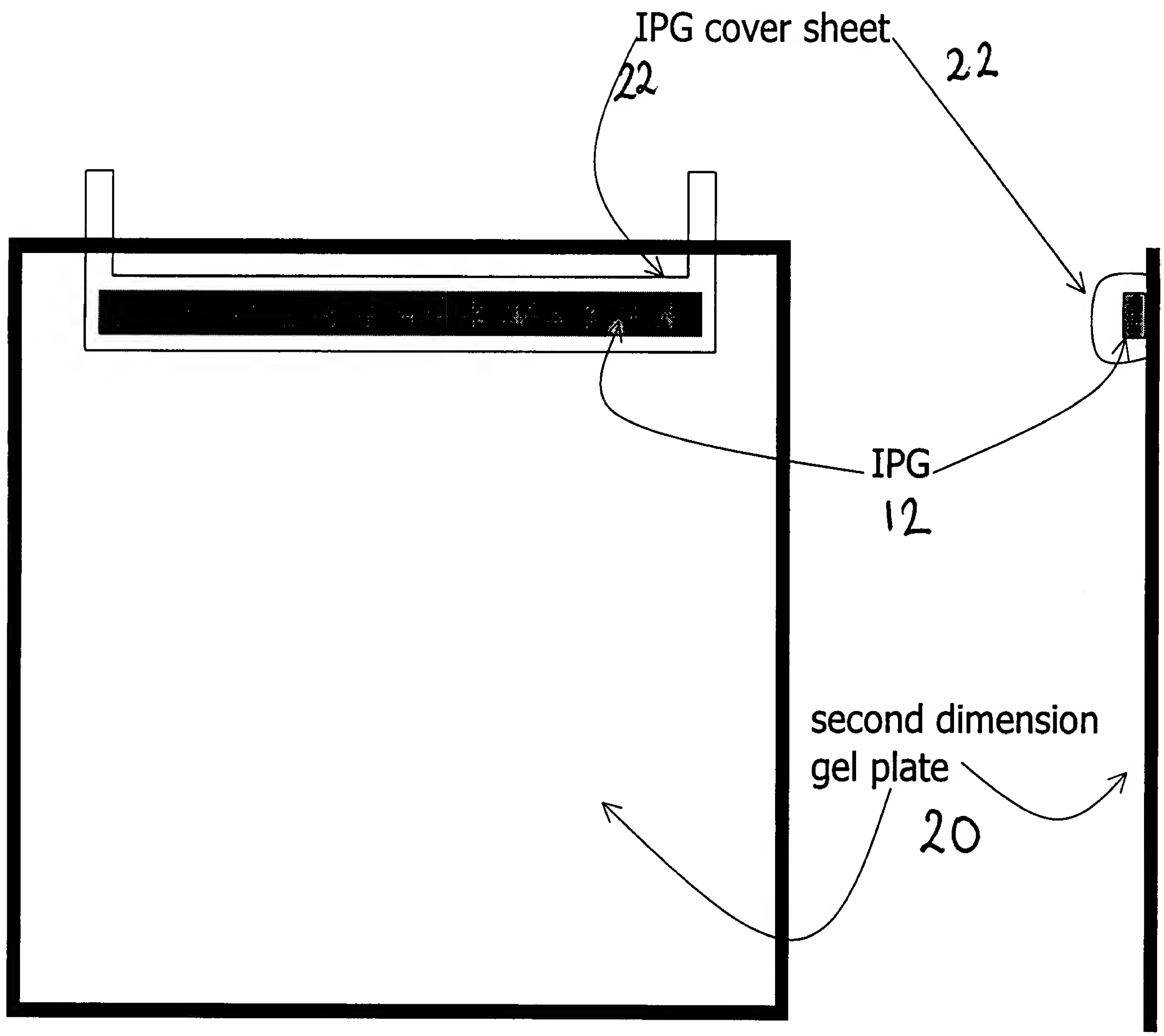

Figure 4

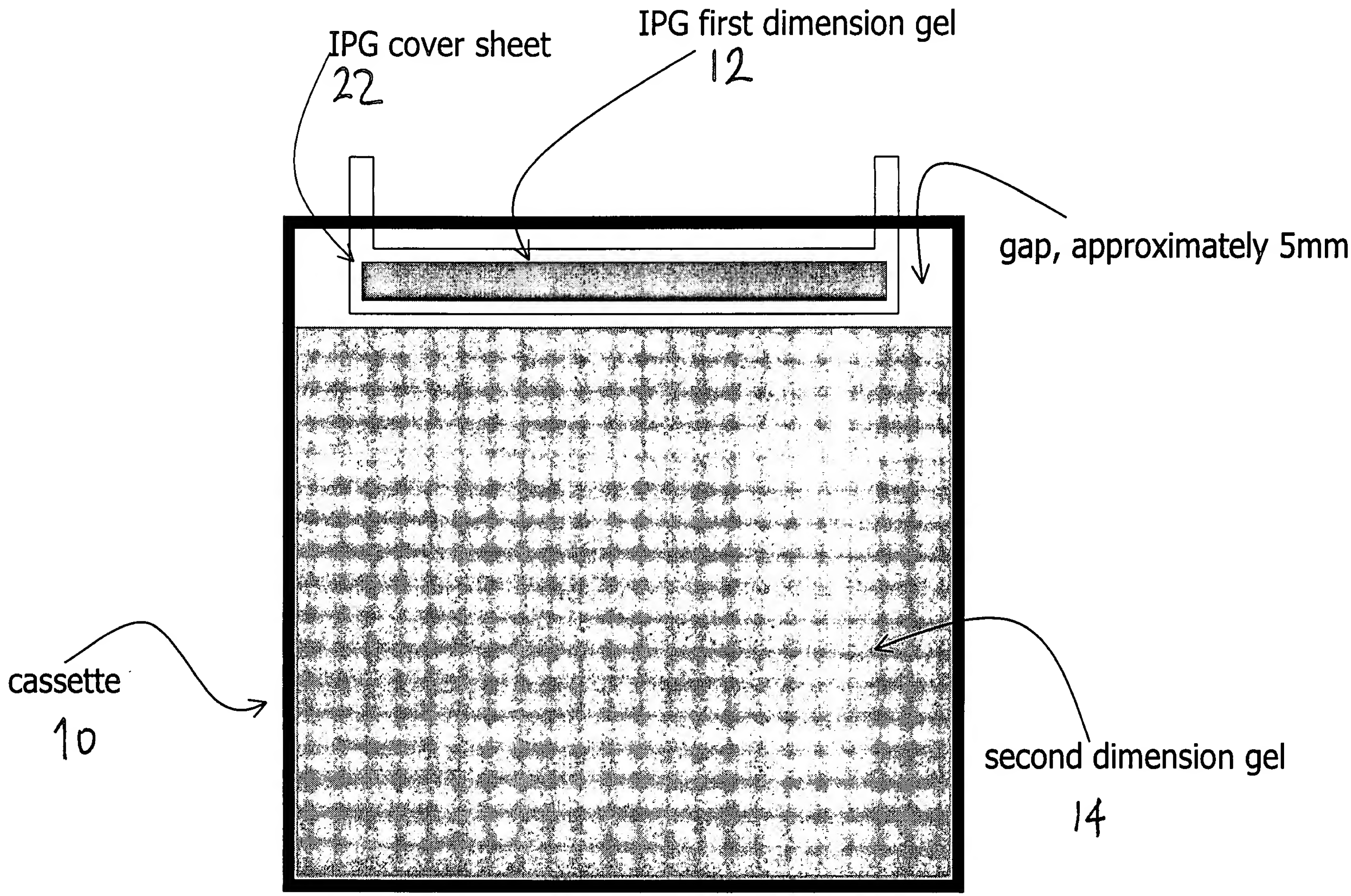
IPG cover sheet
22
IPG first dimension gel
12
gap, approximately 5mm
cassette
10
second dimension gel
14

Figure 5

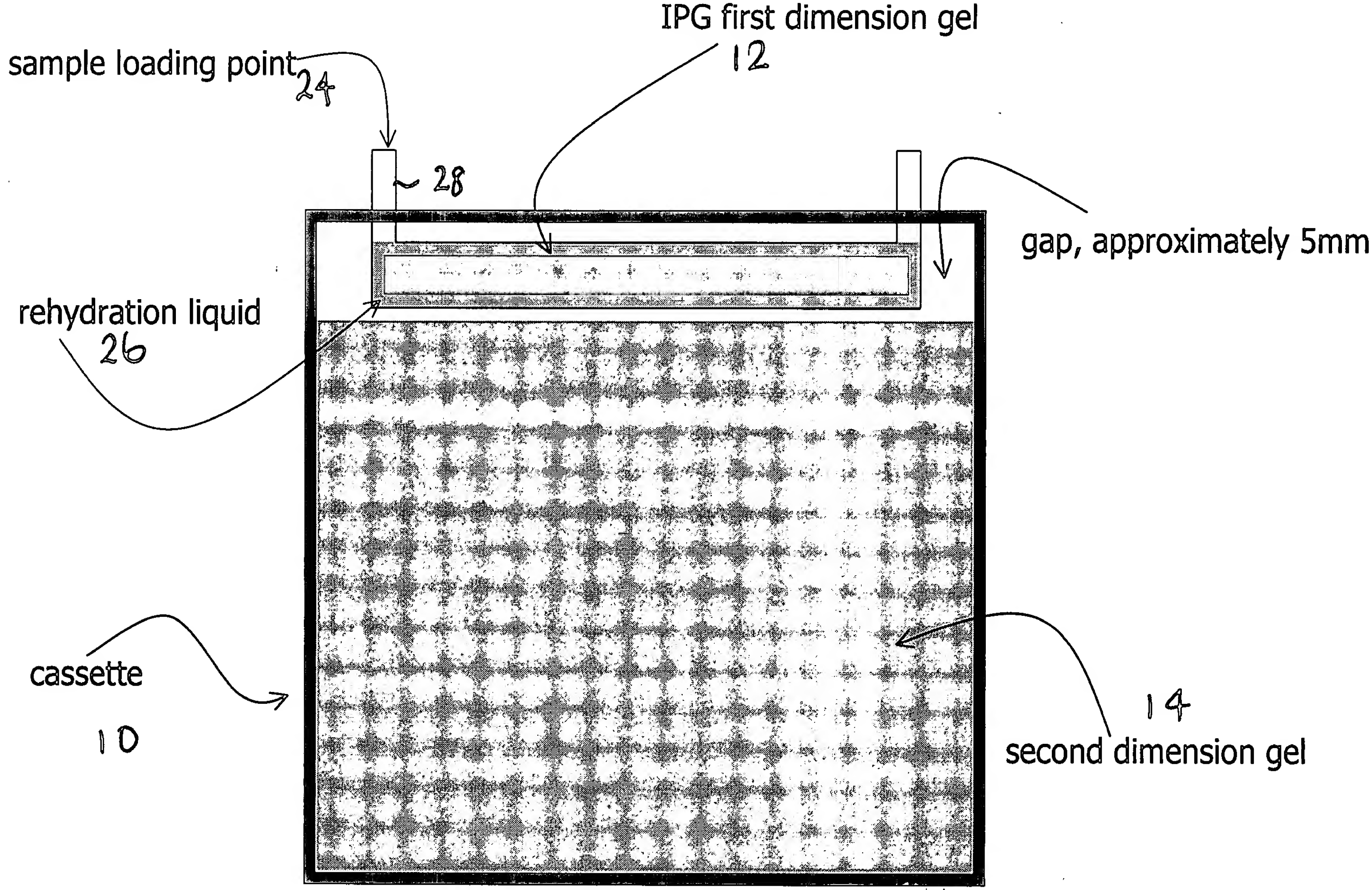
sample loading point 24
28
IPG first dimension gel 12
gap, approximately 5mm
rehydration liquid 26
cassette 10
14 second dimension gel

Figure 6

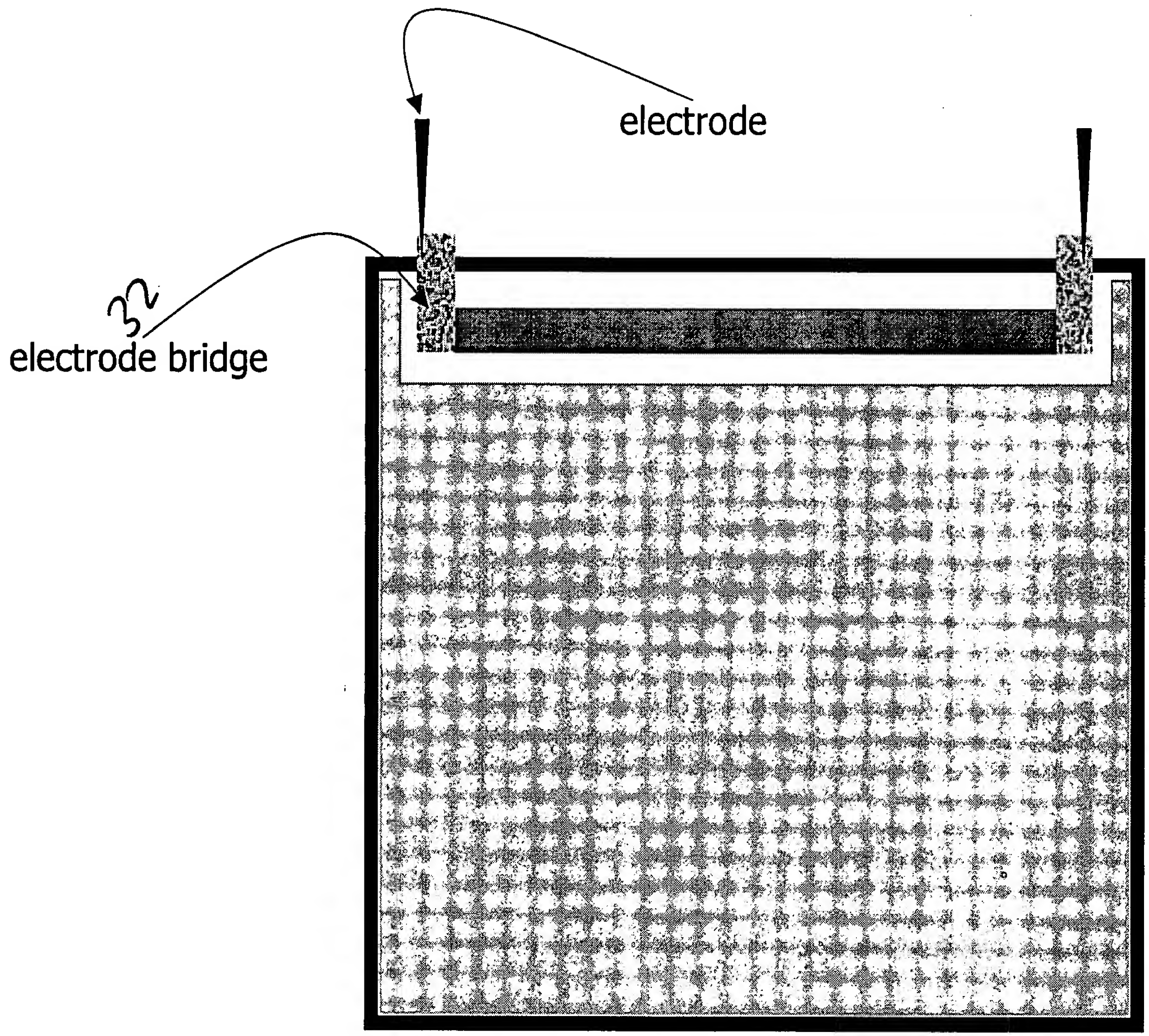

Figure 7

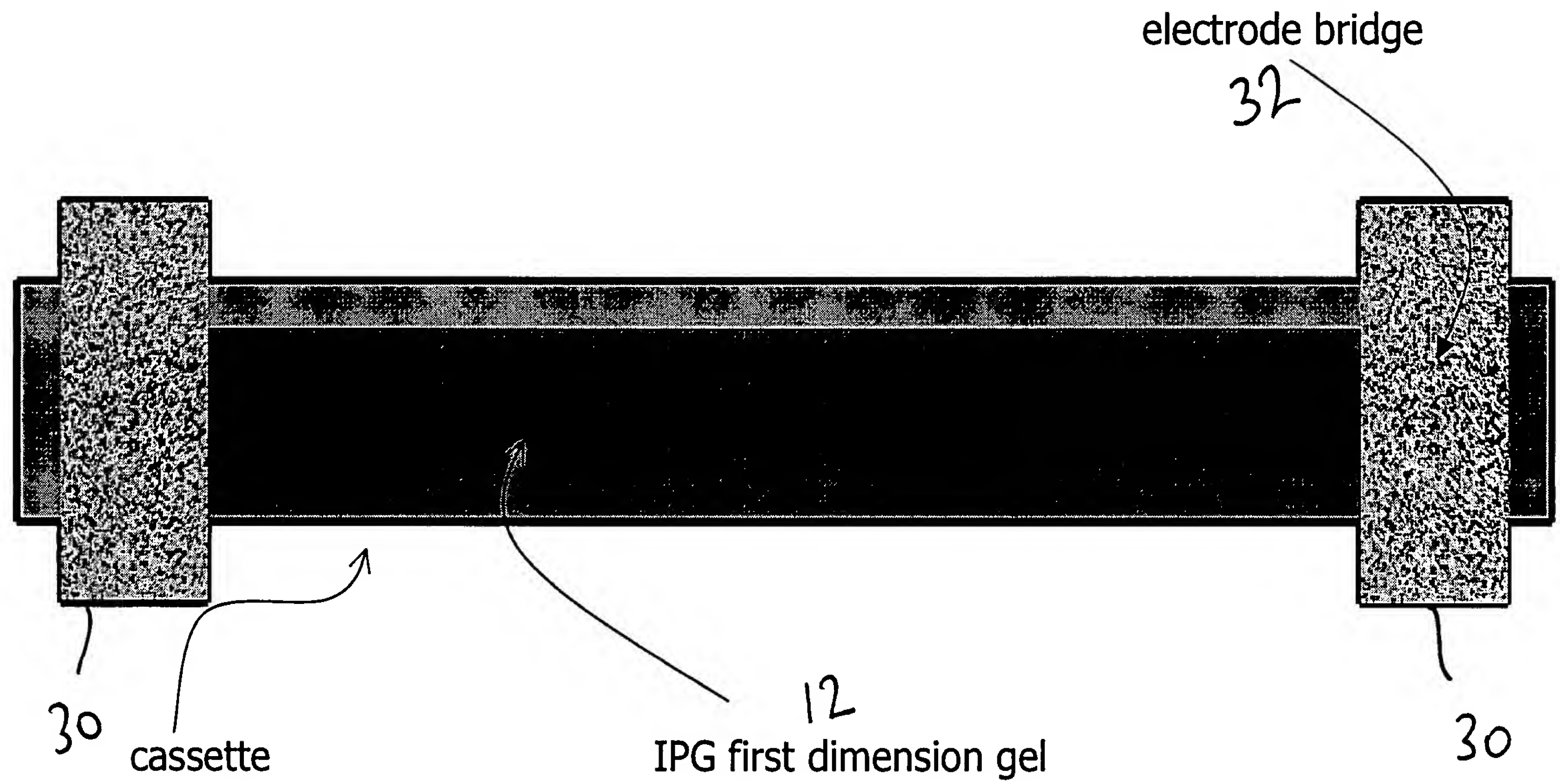
electrode bridge
32
30
cassette
12
IPG first dimension gel
30

Figure 8

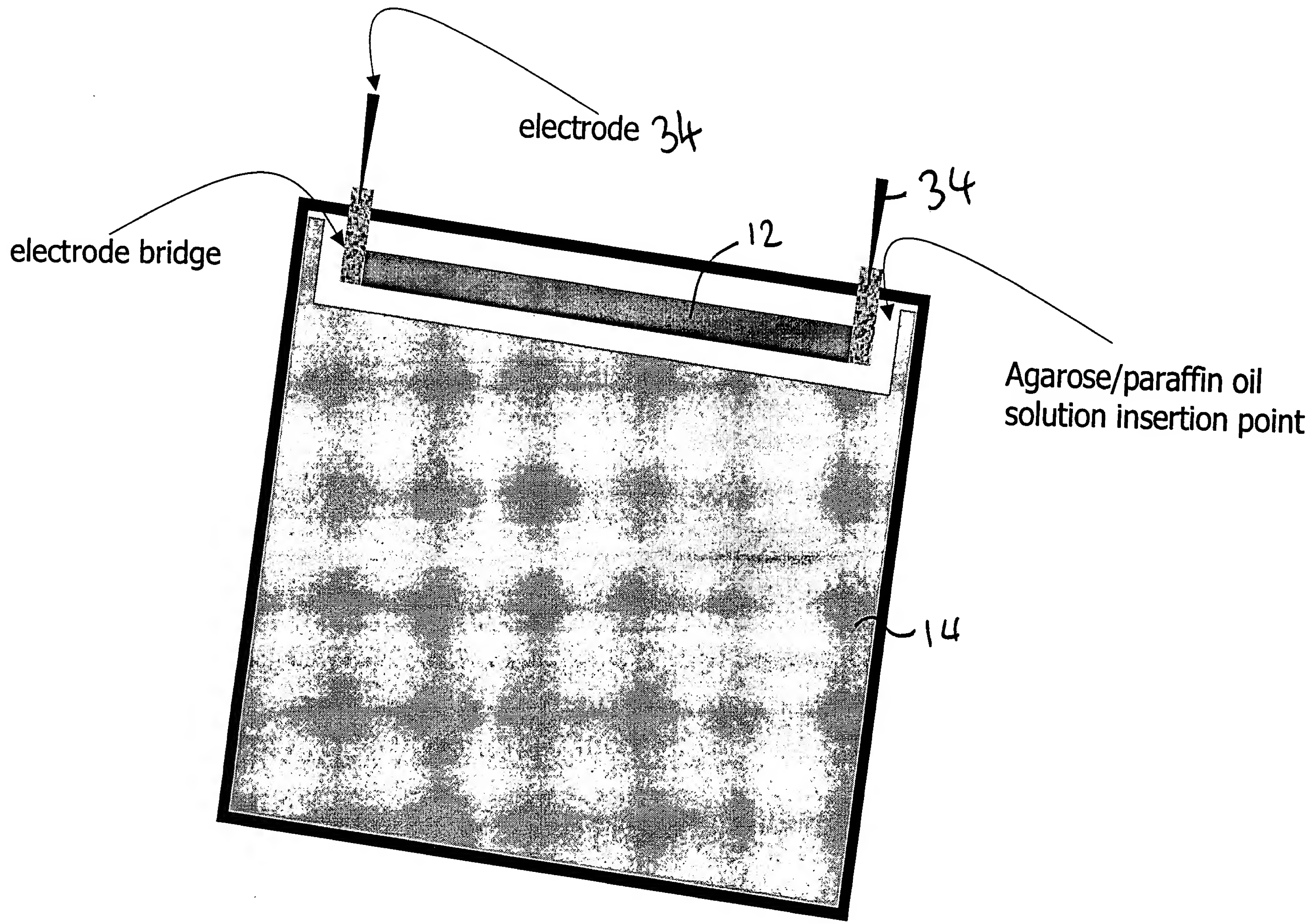
electrode 34
34
electrode bridge
12
Agarose/paraffin oil solution insertion point
14

Figure 9